

Atypical *Aeromonas salmonicida* Isolated from Diseased Turbot (*Scophthalmus maximus* L.)

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Larsen J.L. and K. Pedersen: Atypical *Aeromonas salmonicida* isolated from diseased turbot (*Scophthalmus maximus* L.). Acta vet. scand. 1996, 37, 139-146. – Atypical *Aeromonas salmonicida* were isolated from 3 outbreaks of disease among farmed turbot (*Scophthalmus maximus* L.) in 3 different farms, 1 from Norway (N1) and 2 from Denmark (DK1 and DK2). In all 3 cases, the incidence of disease and mortality was high and the main characteristic pathological finding was skin ulcers and septicaemia. The isolated bacteria were subjected to a thorough phenotypic and genotypic examination and comparison in the laboratory. All 3 isolates belonged to *A. salmonicida* but displayed some very different biochemical properties. However, the 2 Danish strains, DK1 and DK2 had identical ribotypes but different from that of N1, whereas the plasmid profiles of DK1 and N1 were identical but different from that of DK2. These observations emphasize the need for an improvement of our understanding of the taxonomy and epidemiology of atypical *A. salmonicida*.

fish; pathogen; furunculosis.

Introduction

Austin *et al.* (1989) confirmed the validity of the *Aeromonas salmonicida* subspecies listed in Bergey's manual (Popoff 1984) and added a description of a new subspecies *A. salmonicida* subsp. *smithia* to the 3 existing, *A. salmonicida* subsp. *salmonicida*, *A. salmonicida* subsp. *achromogenes*, and *Aeromonas salmonicida* subsp. *masoucida*. However, a review of the literature together with new information (Pedersen *et al.* 1994, 1995, Wiklund *et al.* 1994) suggest that the proposed subspecies failed to encompass many new isolates of atypical *A. salmonicida*. As far as concerns the growth conditions, they appear to separate into a very fastidious, slow growing group and a group growing like "typical" *A. salmonicida* subsp. *salmonicida* (Wiklund & Dalsgaard 1994). In the definition of the genus *Aeromonas*, it is

mentioned that *Aeromonas* occurs in fresh water and sewage (Popoff 1984). However, *A. salmonicida* subsp. *salmonicida* has been transferred with smolt of rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) to sea farms where the organism is a permanent threat to an economical profitable production (Larsen & Møllergaard 1981, Lillehaug *et al.* 1992). *A. salmonicida* can survive for up to 18 months in sediments beneath fish farms (Husevåg & Lunestad 1995) and be transported to the surface, attached to gas bubbles or lipid droplets, and subsequently to the farmed fish (Husevåg 1991, Enger 1992). Studies of flatfishes such as flounder (*Platichthys flesus*), dab (*Limanda limanda*), and plaice (*Pleuronectes platessa*) have indicated that different atypical *A. salmonicida* are involved in ulcer-disease (Vethaak 1992, Wiklund & Bylund

1993, Wiklund & Dalsgaard 1994), and it has been found that such organisms can enter a fish farm producing turbot and cause a septicaemic infection with the occurrence of ulcers and heavy mortality (Pedersen *et al.* 1994).

This paper deals with a characterization of isolates of atypical *A. salmonicida* from 3 outbreaks of disease in turbot farms (1 in Norway and 2 in Denmark) and it also addresses the importance of these organisms from a diagnostic and a forensic point of view.

Materials and methods

Bacterial isolates

Atypical *A. salmonicida* were isolated from pronephros and ulcers of diseased fish from 2 Danish fishfarms (DK1 and DK2) one of which (DK1) was described previously (Pedersen *et al.* 1994), and isolates were received from collaborative work with a Norwegian production unit – N1 (Gravingen & Dyddland 1991). From DK1, 10 strains were included, isolated from turbot of 50–300 g body weight. From DK2, only a single isolate was available. Only one fish was submitted to the laboratory for examination and when requesting more specimens, all fishes had been treated with antibiotics due to high daily mortality. These samples were therefore sterile. The fishes from DK2 were juvenile, 5–10 g. From N1, 4 strains were included, all from juvenile fish, 5–10 g.

The type culture strains *A. salmonicida* subsp. *achromogenes* NCMB 1110 and *A. salmonicida* subsp. *salmonicida* NCMB 1102 were used for comparison.

Bacteriological and serological examination

Initial cultures were made on marine agar (Difco, Detroit, MI, USA), supplemented with 5% calf blood, and incubated at 20°C for 4–7 days. The isolates were characterized according to the principles and by using the methods out-

lined by Dalsgaard *et al.* (1994), while serological examination of *Aeromonas* was performed as described by Dalsgaard & Paulsen (1986).

The presence of an A-layer like compound was determined after principles listed by Pedersen *et al.* (1994).

Protein staining

The reaction of *Aeromonas* colonies with Coomassie brilliant blue and Congo red was examined as described by Evenberg *et al.* (1985) and Ishiguro *et al.* (1985), respectively.

Plasmid profiling

Cultures grown in brain heart infusion broth (-Difco) for 2–5 days (depending on growth intensity) were used for the determination of plasmid profiles. The plasmids were extracted from the bacteria by the method of Kado & Liu (1981). Plasmids were separated by gel electrophoresis in 0.6–1.0% agarose gels (Litex LSL) in TAE (Tris 40 mM, sodium acetate 5 mM, EDTA 1 mM) buffer, pH 8.0.

Ribotyping

Isolation of total DNA was performed according to Pedersen & Larsen (1993). Restriction enzyme digestion was performed as follows: 5 μ l corresponding to 5–10 μ g of the total DNA solutions was digested with the restriction enzyme *Hind*III, (Boehringer, Mannheim, Germany) according to the instruction of the manufacturer. Digested DNA was precipitated with 22 μ l 7.5 M ammonium acetate and 130 μ l 96% ethanol, centrifuged and resuspended in 20 μ l distilled water. Ten μ l loading buffer (EDTA 2 mM pH 8.0, bromophenol blue 0.1%, glycerol 30%) was then added to the resuspended material and the mixture was subjected to electrophoresis in 0.8% agarose gels (Litex LSL) in TAE buffer, pH 8.0. Subsequently, gels were stained with ethidium bromide, 2 μ g/ml (Sigma), and photographed at 254 nm UV tran-

sillumination. DNA fragments were blotted onto nylon membranes (Hybond-N, Amersham) and fixed to the membranes by baking at 80°C for 1 h. Hybridization with a digoxigenin labelled DNA probe complementary to the 16S and 23S rRNA of *E. coli* and subsequent detection of hybridized fragments using alkaline phosphatase labelled anti-digoxigenin (Boehringer) and 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Boehringer) was carried out as previously described (Pedersen & Larsen 1993).

Results

Biochemical investigations

The general properties of the turbot isolates (Table 1) showed that strains from N1 and DK1 were oxidase negative which coincided with a very fastidious growth that was stimulated by the addition of serum. X- or V-factors did not stimulate the growth, nor did elevated CO₂ partial pressure as established by the use of "candle jars". The isolate from DK2 was oxidase positive and grew with an intensity like the 2 type cultures. They were all CAMP negative and produced neither hemolysin nor brown pigment. All atypical *A. salmonicida* from turbot appeared to be very hydrophobic like NCMB 1102 as estimated by their growth in broth and on Congo red and Coomassie brilliant blue agar. They also reacted with an *A. salmonicida* subsp. *salmonicida* antiserum and a purified protein A preparation. Additionally, all turbot isolates were negative in arginine-, lysine-, and ornithine-decarboxylase, arabinose, cellobiose, sorbitol, and lecithin and positive in VP, gelatine, starch, and Tween 80.

The 2 groups of fastidious atypical *A. salmonicida* (N1 and DK1 isolates) behaved similarly in indole, sucrose, mannitol (negative), esculin, salicin, (positive) and were sensitive to ampicillin and resistant to cephalotin. They behaved,

however, differently in glycerol, trehalose, MR, nitrate, where the DK1 and DK2 isolates had identical reactions, and in casein.

The DK2 isolate was oxidase positive, grew on MacConkey agar and differed in the characters indole, esculin, salicin, mannitol and sucrose. In general, the turbot isolates were quite different from the type cultures used for comparison.

Plasmid profiles

The fastidious isolates from Norway and Denmark (DK1) all possessed 3 plasmids of identical sizes 4.6, 7.4 and 78 Kb, respectively, while the isolate from DK2 harbored 6 plasmids of 4.6, 6.2, 16, 34, 45 and 96 kb (Fig. 1).

Ribotyping

The ribotypes of the 3 isolates are shown in Fig. 2. The 2 Danish isolates, DK1 and DK2, had identical ribotypes while the Norwegian strain had a slightly deviating profile.

Discussion

As previously mentioned, there is still some confusion concerning how many taxa should be recognized within *A. salmonicida* and especially within the atypical group. The present study illustrates this problem, as isolates of atypical *A. salmonicida* from 3 separate outbreaks of disease among turbot were caused by strains with quite different biochemical behaviour. From 2 outbreaks – one in Norway and one in Denmark – very fastidious strains were isolated. However, upon repeated isolation, strains from the same outbreak were identical in their reactions, but when comparing strains from the 2 outbreaks, they possessed many different characters. Their growth was neither stimulated by X- and V-factors, nor by elevated CO₂ tension, but serum was necessary for their growth. The second Danish isolate displayed a growth modus similar to that of the type strains, but

Table 1. Biochemical investigation of atypical *Aeromonas salmonicida* isolated from turbot. General properties.

	Strain				
	N1	DK1	DK2	NCMB 1110	NCMB 1102
Gram	-	-	-	-	-
Motility	-	-	-	-	-
Catalase	+	+	+	+	+
Oxidase	-	-	+	+	+
O/F test	F ¹	F	F	F	F
Glucose, gas	-	-	-	-	+
Hemolysin	-	-	-	-	+
Pigment	-	-	-	-	+
Growth in broth	G ²	G	G	U ³	G
Serum needed	+	-	-	-	-
Growth on MacConkey	-	-	+	+	+
Growth at 37°C	-	-	-	-	-
Congo red	(+)	+	+	-	+
Coomassie blue	+	+	+	+	+
Agglutination with <i>A. salmonicida</i> serum	+	+	+	-	+
A-layer	+	+	+	-	+
Arginine	-	-	-	+	+
Lysine	-	-	-	-	+
Ornithine	-	-	-	-	-
Indole	-	-	+	+	-
MR	-	+	+	-	-
VP	+	+	+	+	-
Nitrate	-	+	+	+	+
Casein	+	-	-	+	+
Gelatine	+	+	+	-	+
Lecithin	-	-	-	-	+
Tween 80	+	+	+	+	+
Glycerol	-	+	+	+	+
Arabinose	-	-	-	-	-
Mannitol	-	-	+	+	-
Sorbitol	-	-	-	-	-
Cellulose	-	-	-	-	-
Sucrose	-	-	+	+	-
Trehalose	-	+	+	+	+
Esculin	late +	late +	-	-	+
Salicin	+	+	-	-	+
Ampicillin	S ⁴	S	R ⁵	R	S
Cephalothin	R	R	R	R	S

¹ F = fermentative² G = granular³ U = uniform⁴ S = sensitive⁵ R = resistantAll strains were CAMP negative and not stimulated by X- or V-factor or by increased CO₂ tension.

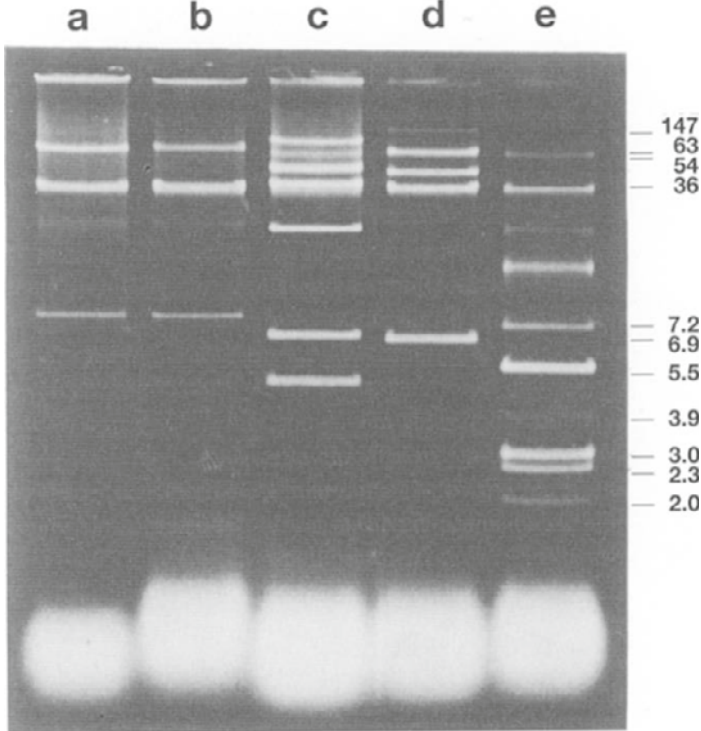


Figure 1. Plasmid profiles of atypical *Aeromonas salmonicida* isolated from turbot. Lane a: strain 91-2-11 from Norway, N1 (4.6, 7.4, and 78 kb); lane b: strain 92-8-149 from Denmark, DK1 (4.6, 7.4, and 78 kb); lane c: strain 92-10-237 from Denmark, DK2 (4.6, 6.2, 18, 34, 45, and 96 kb); lane d: *Escherichia coli* 39R861 size reference plasmid molecules, 6.9, 36, 63, and 147 kb; lane e: *E. coli* V517 size reference plasmid molecules, 2.0, 2.3, 3.0, 3.9, 5.5, 7.2, and 54 kb. The molecular weights of the reference plasmids are marked to the right. The 4.6 kb band of the N1 and DK1 strains is very weak.

none of the present strains could be encompassed by the subspecies dealt with in the literature (Austin & Austin 1993). In general, the turbot isolates shared the properties with *A. salmonicida* subsp. *salmonicida* NCMB 1102 with respect to their reaction with antisera against this species, their growth appearance on Congo red and Coomassie brilliant blue agar as well as in the presence of an A-layer like protein. However, in additional characters, significant discrepancies between the present isolates and the subspecies described in the literature (Austin & Austin 1993) were recorded.

The increasing number of reports dealing with

new isolates of atypical *A. salmonicida* from both fresh water and seawater fish in aquaculture makes this group of bacteria very interesting from an epizootiological as well as a taxonomic point of view. However, the epizootiology and pathogenesis are very poorly understood. It might be suggested that feral fish could be actively infected or act as carriers and in this way constitute a pool from which atypical *A. salmonicida* could be transported to different aquaculture systems, infect sensitive populations of fish, and subsequently spread in the system. There is no doubt about the importance of flatfishes as a reservoir for atypical *A. salmo-*

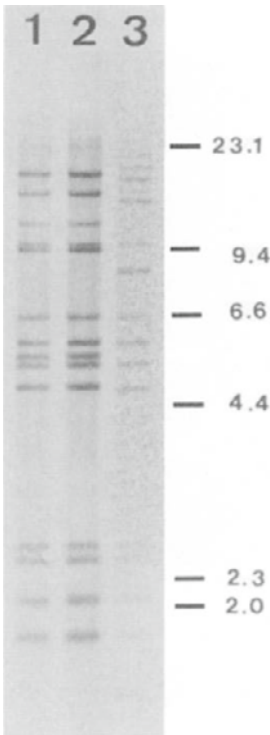


Figure 2. Ribotypes of atypical *Aeromonas salmonicida* isolated from turbot. Lane a: Strain 92-10-237 from Denmark DK2; lane b: strain 92-8-149 from Denmark DK1; lane c: strain 91-2-13 from Norway N1. The molecular weight markers of *Hind*III digested λ DNA are shown to the right: 2.0, 2.3, 4.4, 6.6, 9.4, and 23.1 kb fragments.

nicida, as skin ulcers in flatfishes are caused by these organisms (Wiklund & Dalsgaard 1994), and it might be suggested that the 3 outbreaks reported in the present paper were caused by bacteria transported to the aquaculture facilities by the water intake. Genotypic characterization of atypical *A. salmonicida* isolates has shown that they are different in different waters (Wiklund & Dalsgaard 1995), an observation that also supports the hypothesis that these infections are caused by local strains. Atypical *A. salmonicida* is the only group of *A. salmonicida* causing infections in Iceland (Laxdal 1989),

and in Norway they seemed to occur with increasing frequency before the introduction of fish vaccines with oil adjuvants (Steinar Høie, personal communication). It should be emphasized that despite the fact that these atypical *A. salmonicida* share many pathogenic properties with *A. salmonicida* subsp. *salmonicida*, their epizootiological behaviour is quite different, and they should be regarded differently also from a forensic point of view.

In a recent study, the plasmid profiles and ribotypes of a group of atypical *A. salmonicida* were investigated (Pedersen *et al.* 1996). All these strains contained plasmids and almost all strains carried small as well as large plasmids, comparable in size to those detected here from DK1, DK2, and N1 strains. The study of Pedersen *et al.* (1996) indicated that plasmid profiles as well as ribotypes were very diverse among atypical *A. salmonicida* strains. In contrast, several investigations have shown that plasmid profiles as well as ribotypes of *A. salmonicida* subsp. *salmonicida* are very homogeneous (Bast *et al.* 1988, Nielsen *et al.* 1993, 1994). The study of Pedersen *et al.* (1996) showed that plasmid profiles could change during an outbreak, indicating that plasmids of atypical *A. salmonicida* can be labile whereas ribotypes were more stable. It is therefore remarkable that the ribotypes of the 2 Danish strains in the present investigation were identical but different from the Norwegian strain, while the plasmid profiles of one Danish isolate was identical to the profile of the Norwegian strain, but different from the other Danish strain. However, the ribotype profiles were similar to other profiles reported in the literature (Pedersen *et al.* 1996) and likewise, all isolates contained one or more large plasmid and one or more smaller ones, also in accordance with previous reports (Bast *et al.* 1988, Pedersen *et al.* 1996).

We conclude that the present status of our knowledge of the importance of the atypical *A.*

salmonicida still requires a comprehensive study of the taxonomy and ecology of these organisms.

Acknowledgments

This work was supported by The Danish Agricultural and Veterinary Research Council, grant no. 13-4710-1. The technical assistance of Mrs. M. Højgård is gratefully appreciated.

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Sammendrag

Atypiske *Aeromonas salmonicida* isoleret fra syge pighvarre (*Scophthalmus maximus*).
Atypiske *Aeromonas salmonicida* blev isoleret fra 3

sygdomsudbrud hos pighvar (*Scophthalmus maximus* L.) opdrættet i 3 forskellige farme, 1 fra Norge (N1) og 2 fra Danmark (DK1 og DK2). I alle 3 tilfælde var både incidensen og mortaliteten høj, og de mest karakteristiske fund var ulcerationer i huden samt septikæmi. De isolerede bakterier blev underkastet en grundig fænotypisk og genotypisk undersøgelse i laboratoriet. Alle 3 isolater hørte til de atypiske *A. salmonicida*, men udviste en række meget forskellige biokemiske reaktioner. Det viste sig imidlertid, at de 2 danske isolater, DK1 og DK2 havde identisk ribotype, som var forskellig fra N1's ribotype, mens plasmid profilerne for DK1 og N1 var identiske, men forskellige fra DK2's profil. Disse observationer understreger behovet for en forbedring af vores forståelse af de atypiske *Aeromonas salmonicida* isolaters taxonomi og epidemiologi.

(Accepted December 16, 1995).

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